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Review

Bringing order to a complex molecular machine: The assembly of the bacterial flagella

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Abstract

The bacterial flagellum is an example of elegance in molecular engineering. Flagella dependent motility is a widespread and evolutionarily ancient trait. Diverse bacterial species have evolved unique structural adaptations enabling them to migrate in their environmental niche. Variability exists in the number, location and configuration of flagella, and reflects unique adaptations of the microorganism. The most detailed analysis of flagellar morphogenesis and structure has focused on *Escherichia coli* and *Salmonella enterica*. The appendage assembles sequentially from the inner to the outer-most structures. Additionally the temporal order of gene expression correlates with the assembly order of encoded proteins into the final structure. The bacterial flagellar apparatus includes an essential basal body complex that comprises the export machinery required for assembly of the hook and flagellar filament. A review outlining the current understanding of the protein interactions that make up this remarkable structure will be presented, and the associated temporal genetic regulation will be briefly discussed.

Keywords: Flagella; Motitlity; Basal body; Chemotaxis

Contents

1. Introduction

Flagellar dependent motility is widespread in bacteria however the number, location, and structure of the flagellar apparatus has evolved different features in individual species reflecting unique adaptations in those organisms. The most detailed analysis has been in *E. coli* and *S. enterica* and will be the focus of this review. For four decades researchers have

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Table 1
Experimental strategies employed to investigate the protein interactions of the bacterial flagella

Strategy	Key references
Electron (cryo)microscopy	[4–7,30,49–51,62,66,69]
Yeast two-hybrid system	[10]
Sedimentation	[19]
Affinity blotting/chromatography	[3,11,18,20,29,39,54,60,79]
Protein fusion	[7,14,34,35,44,45,84,85]
Motility studies	[5,17,21,38,42,71,72,86]

employed inventive strategies to characterize this fascinating system responsible for bacterial motility and chemotaxis (Table 1). The bacterial flagellum comprises a complex membrane spanning organization encompassing numerous protein interactions. It is the complex of these proteins that is a requisite for the sequential assembly from the most proximal to the distal structures of the mature flagellum. Typically three components are discussed in the assemblage of the structure: the flagellar rod, the hook, and the filament. We will discuss these components in some detail and provide additional insight into the C and MS-rings; the machinery from which the organization begins, and that is indispensable for the function of the completed apparatus.

2. Protein interactions of the C and MS-ring

Flagellar assembly begins when FliF, an integral protein that comprises the MS-ring, is incorporated into the bacterial cytoplasmic membrane [1]. This process is dependent on the Sec export mechanism [2]. The MS-ring is the starting point from which all other flagellar structures will be assembled [3]. FliF assembles into a single ring that appears as two adjacent loops that span the cytoplasmic membrane [4]. Cryomicrosopy coupled with the 3D reconstruction of the MS-ring reveals the structure has 24–26 fold symmetry [5]. The inner-loop functions as part of the proximal rod, while the outer loop is the thick edge portion of the MS-ring. The central channel of the MS-ring exists in two distinct confirmations. The N-terminus of FliF is thought to act as the plug that exists in the membrane channel [6]. This suggested that FliF itself may occupy a central core of the MS-ring and act as an export/gate mechanism, whereby the proximal molecular environment dictates whether the plug is open or closed [4]. In fact, it is suggested that the presence of this central plug allows cells to remain viable even when *fliF* is over-expressed [1]. Alternatively, the MS-ring may trap a disc of cytoplasmic membrane that effectively blocks the pore.

Once FliF is incorporated into the membrane, FliG binds the cytoplasmic face of the MS-ring [7], and later FliM and FliN are recruited to the membrane bound structure [3]. It has been established that this is the sequence of events in initial assembly of the MS-ring. Mutants of FliM, FliN and FliG as well as proteins involved in the flagellar type III secretion system (fTTSS) still assemble an MS-ring [8]. Immunoblotting experiments have established that FliM, FliN and FliG require FliF to be incorporated into the cytoplasmic membrane. FliG does not need FliM or FliN, however FliM and FliN cannot associate with the membrane without FliG [3]. Only 9 amino acids at the C-terminus of FliF are essential for flagellar assembly [9]. Marykwas et al. carried out a two-hybrid screen to investigate the protein interactions in early flagellar morphogenesis [10]. The experiments demonstrated interaction between FliM–FliG, FliM–FliM and FliM–FliN. Subsequent screening of a protein fusion library showed direct contact of FliF–FliG. The interactions were investigated further and it was recognized that 52 C-terminal amino acids of FliM were required for contact with FliN, but were dispensable for the FliM–FliG interaction [10]. An unexpected interaction between FliF and H-NS (histone-like protein) was also discovered indicating an additional level of complexity in the assembly process [10].

The C-ring is the only flagellar structure that is entirely confined to the bacterial cytoplasm. It comprises the most proximal organization of the bacterial flagellar complex and is composed of numerous proteins. Within a functional flagellum the role of rotation and switching have been attributed to the C-ring [11]. FliG comprises the upper part of the C-ring, and connects the C-ring to the MS-ring [12–14]. FliG is one of the earliest proteins added during basal body assembly [15], and forms a close interaction with FliF. Strains that contained a FliF–FliG fusion were motile and underwent rotational switching [7] suggesting the two proteins are intimately associated in wild type cells. FliG residues 183–196 constitute a flexible domain between N-terminal assembly and C-terminal motility domain [15]. The FliG protein has a conserved surface patch $EHPQ_{125-128}$ and R_{160} as well as a C-terminal hydrophobic patch. Residue mutations in these regions disrupted flagellar assembly or introduced rotational biases in flagellar function [11]. Meanwhile mutations in the flexible domain (residues 183–196) had a minimal influence on flagellar function [15].

Approximately 32–36 FliM monomers interact with the FliN tetramer [12] via its C-terminal domain [10,16]. FliM/FliN function as the rotor/switch proteins in the mature complex [2]. FliM is positioned between FliG and FliN and has been shown to interact with both proteins [10]. The exact mode of assembly of the C-ring has remained elusive, however Brown et al. have proposed a model where 26 FliM residues bind the hydrophobicity patch on the C-terminal domain of FliG, while the remaining 8 monomers are tilted to interact with the EHPQR residues of FliG [11]. This was an important interaction to establish, as it concluded that that the C-ring has the same symmetry as the MS-ring [11,12]. The 26 monomers of FliM interact with 26 FliG monomers that in turn are interacting with 26 monomers of FliF that make up the MS-ring. This proposed symmetry has been confirmed by cryomicroscopy [5].

The functional domains of FliM have been extensively investigated and it has been established by affinity blotting and characterization of various *fliM* mutants that the N-terminus of FliM binds CheY∼P and is involved in rotational switching, the middle region binds FliG and is involved in switching/stator rotation, and the C-terminus of FliM interacts with FliN and is involved in flagellar assembly [17,18]. Brown et al. [19] used equilibrium sedimentation of FliN to establish a stoichiometric ratio of $FliM_1FliN_4$. FliN has conserved hydrophobic residues on the surface of the protein and mutation in the hydrophobic

domains affected both assembly and switching [19]. FliN is the most abundant protein in the flagellar basal body, with 110 monomers present in the C-ring structure [2]. FliN directly interacts with FliH; a components that regulates the activity of the fTTSS ATPase (FliI) [20]. Null mutants of *fliN* failed to assemble flagella and it was supposed that in addition to motility, FliN played some role in export of flagellar component proteins [21].

3. Proteins of the rod, the L and P rings and the fTTSS

The basal body spans the cytoplasmic and outer-membrane acting as a relay of rotational force from the motor to the hook apparatus. The rod is a major component of the flagellar basal body and spans the bacterial periplasm with its simple yet elegant architecture. The rod components are exported across the cytoplasmic membrane by the fTTSS [2,22]. The rod must be tightly associated with FliF so as to efficiently transmit the generated torque to the hook [23]. Five proteins make up the flagellar rod: FlgB, FlgC, FlgF, FlgG and FliE. The amino acid sequence homology at the terminal ends of these distinct proteins proposes that that they assemble into a helical organization of subunits [23]. Each axial protein comprising the rod possesses a heptad terminal sequence of hydrophobic amino acids suggesting that the rod assembles into a coiled-coil conformation [24]. FlgG lies on the distal portion of the rod, just before the rod-hook junction, and FliE is postulated to be the proximal component of the rod, located near the MS-ring/rod junction [2,3]. The precise order of the other rod subunits is not known [24].

Proteins involved in flagellar export share sequence homology to proteins of type III secretion systems [25]. Type III export is characterized by the absence of a conserved amino acid signal sequence on the secreted protein, however a disordered N-terminal domain is required for secretion [26]. Export of proteins is catalyzed by an ATPase that participates in chaperone release, and partial unfolding of the export protein substrate [27]. All exported proteins except for FlhP (P-ring) FliH (L-ring) and FlhA (chaperone) are translocated via the fTTSS [28].

Minamino and Macnab [29] designed a simple experiment to determine if mutants deficient in putative export fTTSS proteins could export bacterial flagellar substrates. The investigators determined if the substrate could be translocated across the inner-membrane and into the bacterial periplasm. Proteins that were successfully exported were detected in the periplasmic fraction by an immunoblot. Since fTTSS can be further classified into either rod-hook specific or filament specific, temperature sensitive mutants were employed to establish which proteins were necessary for the export of filament-type subunits. FlgD (hook-capping protein) and FlgE (hook protein) required FlhA, FlhB, FliH, FliI, FliO, FliP, FliQ and FliR for export, while FliC (filament protein) required only FlhA, FlhB, FliH, FliI and FliO for membrane translocation [29].

While the N-terminus of FliF may act as a central plug of the MS-ring [6], the proteins FliP and FliR are two additional factors that may facilitate export through the channel by naturally residing in inner-surface of the MS-ring [30]. It has been proposed that all the proteins involved in the fTTSS are housed within a 75-Å central pore [4] of the MS-ring [29]. In fact residues 50–210 of FliF share sequence homology with type III export proteins [6]. The essential functions of the fTTSS are carried out by three cytoplasmic proteins (FliH, FliI, and FliJ), and six integral membrane proteins (FlhA, FlhB, FliO, FliP, FliQ and FliR) [2]. The interesting feature is that the six integral components involved in fTTSS are themselves inserted into the membrane by the Sec pathway [22].

The N-terminal amino acids of FlhA prior to the first transmembrane loop as well as the Phe–His–Ile–Pro–Glu–Pro conserved domain within the cytoplasmic loop between transmembrane domain-4 (TM4) and TM5 which are essential for protein export [31]. Affinity blotting experiments have demonstrated there is an interaction between FlhA and FliF [31], as well as the cytoplasmic domains of FlhA ($FlhA_C$) and the cytoplasmic domain of FlhB ($FlhB_C$). The $FlhA_C$ may interact with $FlhB_C$ and the large C-terminal cytoplasmic domains further act as a docking site for the FliH, FliI and FliJ complex [22,29,32]. Substrate translocation across the cytoplasmic membrane may be facilitated by the ATPase; FliI. Affinity chromatography has implicated a strong interaction between $FlhA_C$ and FliH/FliI [32]. The proteins FliO, FliP, FliQ and FliR do not encode any structural components, but are necessary for flagellar formation [33]. The proteins are likely involved in the fTTSS as they are membrane bound, and have homology with proteins involved in type III secretion in virulent bacteria [28]. An engineered fusion of FliR–FliB remains functional even [34] and a natural FliR–FliB fusion has been described in *Clostridium* [35]. This suggests a 1:1 ratio between the two proteins, and furthermore may indicate an intimate interaction between the fTTSS export components.

The FliH protein forms a heterotrimeric complex with FliI ($FliH_1FliI_2$), and the C-terminal region of FliH is involved in the interaction with the N-terminal region of FliI [22,36]. FliI functions as the ATPase that drives the fTTSS, and has sequence similarity to the F_0F_1 ATPase. Mutations near the N-terminus of FliI blocked flagellar assembly [37], and suggest that a stable FliI–FliH interaction is important for functional ATPase activity. However, FliH may not be essential as a *fliH* null mutants are slightly motile, and overproducing FliI improves motility in the mutant strain [38].

The P-ring is a periplasmic formation that connects with the peptidoglycan cell wall [39]. The P-ring is a homopolymeric structure that is assembled around the flagellar rod, and is comprised of 26 FlgI monomers [28]. FlgI only formed a weak interaction with itself in the yeast two-hybrid system [10], and it has been suggested that FlgA may be required to form a closer contact between the individual monomers of FlgI. This is consistent with the observation that both FlgA and FlgI are required for P-ring formation [39]. FlgA is believed to be a chaperone of FlgI and both proteins are exported across the cytoplasmic membrane via the Sec pathway [2,39]. The chaperone may facilitate proper aggregation of the FlgI monomer into its mature polymerized form.

The C-terminal domain of FliJ is essential for L-ring and hook assembly but is not needed for P-ring formation [40]. Dijkstra

and Keck [41] demonstrated that FliJ has a muramidase motif at the C-terminus, and may be involved in peptidoglycan hydrolysis. Furthermore, FliJ acts as a rod-capping protein and is displaced from the basal body once flagellar hook assembly begins [2]. Therefore, while FlgI can self assemble into the P-ring with the help of FlgA, proteins that make up more distal flagellar structures require a peptidoglycan-hydrolyzing enzyme to transverse the cell wall. The L-ring is made up of 28 monomers of FlgH [2,42]. FlgH is a lipoprotein with lipoylation occurring at the N-terminus of the protein. A mutated *flgH* that coded a protein unable to undergo lipoylation did not inhibit motility when it was overproduced [42]. This suggests that lipoylation of the protein facilitates a more efficient incorporation into the outer-membrane, but is not essential for a functional L-ring formation. The L and P rings interact to form the L/P ring complex. Truncation of the C-terminus of FlgH adversely affected motility, and it has been proposed that the P- and L-ring interact via the C-terminal domain of FlgH [42].

4. Hook assembly and substrate switching in the fTTSS

The hook provides an important function in the motility of the bacteria. The architecture of the hook enables it to act as a flexible hinge relaying the energy generated by the motor into torque formation that is transferred onto the rigid filament [43]. The hook develops to a length of approximately 55 nm, but slight variation exists in the span of the completed structure [44,45]. The hook is composed of monomers of FlgE that form into α-helices upon polymerization [46]. The polymerization is due to hydrophobic heptad repeats at the N and C terminus of the monomeric polypeptide [47]. These terminal residues allow the hook to self assemble from the proximal to the distal end [48]. The completed hook is composed from 120 copies of FlgE [2,49], and X-ray crystallography and electron microscopy data indicate that the hook forms a twisted tubular structure [49] which contains a 25-Å channel running down the center [50].

As soon as hook assembly begins, the rod cap (FlgJ) is displaced by the hook capping protein FlgD [2]. FlgD is believed to induce the initiation of hook assembly [51], but is lacking from the completed flagella [8]. Two putative chaperone proteins of the fTTSS are believed to facilitate the passage of FlgD through the basal body inner-channel. Affinity blotting demonstrated that FlgL and FliT bind FlgD; the hook capping protein [52]. Null mutants of *flgD* secreted FlgE monomers into the supernatant of the culture medium [51]. This implies that while FlgD is not necessary for FlgE export, it serves some function in polymerization of subunits into an α-helical structural arrangement. Monomers of FlgE are exported across the cytoplasmic membrane by the fTTSS. Protein subunits pass through the central channel and self-assemble endogenously from base to tip to form the hook of the flagella. The FlgD hook-cap remains associated at the distal end of the hook during the entire assembly process.

One of the most fascinating questions that presents itself during hook assembly is how the export apparatus knows when the hook has reached an appropriate length. The completion of assembly of early flagellar substrates leads to a switch in export specificity to late flagellar substrates. The substrate specificity changes as a result of the fTTSS protein FlhB and the change is mediated by FliK; the hook-length control protein [43,53–56]. The fTTSS undergoes an irreversible switch in specificity from rod/hook type export substrates to filament type export substrates [29,57,58].

The cleavage of $FlhB_C$ is necessary for the class switching to occur. The cytoplasmic domain of FlhB can be further subdivided into $FlhB_{CN}$ (211–269) and $FlhB_{CC}$ (270–383) that comprise two unique subdomains on the cytoplasmic C-terminus of the integral membrane protein [59]. Minamino and MacNab [60] demonstrated that a cloned sequence of $FlhB_C$ was unstable and underwent cleavage into two subdomains. The cleavage was specific to Pro270, and did not require any proteases or FliK to be present [53]. This indicates that FlhB has an autocleavage mechanism. Furthermore, column chromatography experiments demonstrated that even after cleavage the two subdomains interact with one another [60].

FliK is a 405-amino acid protein, and has been implicated in the interaction with FlhB to induce class switching (from hook to filament assembly). Null mutants of *fliK* produced long polyhooks with no filament [58]. FliK is itself a rod/hook type protein, and its export by the fTTSS prior to the completion of hook assembly [54]. It is the N-terminal domain that is likely to be involved in protein export of FliK, however the C-terminal domain interacts with $FlhB_C$ and may play a role in substrate specificity switching [44]. Interestingly the recent findings of Shibata et al. [56] demonstrate that FliK is able to control hook length even in the absence of its secretion.

Two models currently exist that address the mechanism of substrate switching by FlhB and the role FliK: the cup filling model [61], and the tape measure model [55]. Although the mechanisms will not be discussed in this section, they essentially propose that FliK relays the status of hook assembly to FlhB, so that irreversible substrate specific switching occurs only after enough FlgE monomers have been exported.

5. Hook associated proteins and filament assembly

As the hook attains a mature length, the hook cap is discarded, and replaced by three hook-associated proteins (HAPs) [2]. These are among the first filament type substrates to be exported by the fTTSS. FlgK, FliD and FlgL correspond to HAP1, HAP2 and HAP3, respectively. All three HAPs were detected in the hook-filament of flagellated strains [62]. Specific antibodies for each of the HAP proteins conjugated to gold particles demonstrated that HAP1 and HAP3 were localized at the hook-filament junction [63]. Only because FliD is attached to the hook prior to filament formation is it referred to as HAP. In actuality FliD/HAP2 is present on the tip of the filament in the fully assembled flagellum [62–64]. Stoichiometric analysis of junction proteins reveals 11 FlgK, 11 FlgL and 5 FliD, monomers in the assembled filament [65]. FliN and FliT are chaperones of the filament-type proteins FlgK, FlgL and FliD. FliJ interacts with the chaperone–substrate complex, and may recruit the HAP substrates to the export apparatus located at the cytoplasmic membrane [65]. FliD is a pentagonal disc that

rotates as the monomers of FliC are incorporated into the filament. The filament grows at the tip, and the cap is guided along the helical spiral as filament subunits are incorporated [66].

The flagellar filament is a long helix composed of up to 20,000 FliC subunits [2]. In a comparison of amino acid sequence between different bacterial species, the surface domains of the filament are not conserved, but sequence that mediates filament assembly is highly conserved among different bacteria [67]. The N and C terminus of FliC have conserved hydrophobic residues that interact through a coiled coil interface. The bacterial flagellum can be 15 μm in length with a diameter of only 120–150 Å [68]. Yonekura et al. [69] have solved the structure of the flagella filament by cryomicroscopy, and have concluded the diameter of the central channel is only 20 Å. This imposes certain limitations on the assembly of FliC, and it has been speculated that FliC must be considerably unfolded to pass through the central channel and assemble at the tip of the filament. Conversely the narrow channel may serve some function as it would prevent premature folding of the monomer prior to FliC reaching the tip of the assembling filament [69]. FliS, a chaperone of FliC, binds the C-terminal domain of the filament monomer. *In vitro* experiments have demonstrated that FliC binds FliS and the substrate chaperone interaction prevents polymerization of FliC [70].

6. The stator of the flagellum

Torque generation is driven by a membrane gradient of H^+ in neutrophiles [71,72], and by Na+ in marine *Vibrio* and alkalophiles [73,74]. Mot A and Mot B form the flagellar stator; the non-rotating component of the flagellar motor. MotA/MotB are integral membrane proteins [75], and participate in the initial step of torque generation which is necessary for the rotation of the completed flagella [76]. Mot mutant of *Escherichia coli* produce fully assembled flagella that are incapable of rotation, and are paralyzed [77]. Blair and Berg [78] designed an experiment in which they induced expression of MotA or MotB from a plasmid encoding either Mot protein. When tethered mutants were induced to express the complemented Mot protein, the cells began to rotate around the point of attachment. Over time as the Mot protein levels increased the rotation speed increased in a series of eight sequential steps. From these observations it was concluded that up to eight torque generating units exist in the functional stator, and furthermore this structure is composed of both MotA and MotB [78]. Since these earlier studies, gel filtration chromatography has demonstrated a $MotA_4MotB_2$ stoichiometry of the stator [79]. The mechanisms by which the stator and motor convert ion gradients into torque are beyond the scope of this review. We refer the reader to other literature that should provide several attractive models addressing this biophysical process [80–83].

Alkaline phosphatase protein fusions demonstrated that the carboxyl-terminal portion of MotB is located on the periplasmic side of the inner membrane [84]. An Asp32 residue of MotB located near the inner face of the cytoplasmic membrane is likely involved in proton translocation. This residue forms a probable proton binding site, however the residue's function cannot be replaced by another protonatable amino acid [85]. MotA has a much larger cytoplasmic domain than MotB [76]. Mutagenesis of the MotA cytoplasmic residues Arg90 and Glu98 and the residues at the cytoplasm–membrane spanning interface, Pro173 and Pro222, were determined to be important for MotA function [86]. Motility assays demonstrated that mutations that reversed the charge of the residue were more severe then mutations that neutralized the charge. It is postulated that these charged residues may facilitate an electrostatic interaction with the motor protein FliG [86].

7. Regulation of flagella gene expression

Under particular environmental conditions, *E. coli* synthesizes multiple flagella which facilitate propulsion and chemotaxis. The genes involved in the generation of flagella and chemotactic machinery form an intricate genetic network that includes examples of both positive and negative regulation, as well as regulated feedback loops (Fig. 1). Elegant genetic studies have shown that the 14 flagella operons are arranged in a regulatory cascade of three classes [87–90]. The class 1 operon encodes the transcriptional activator of class 2 operons,

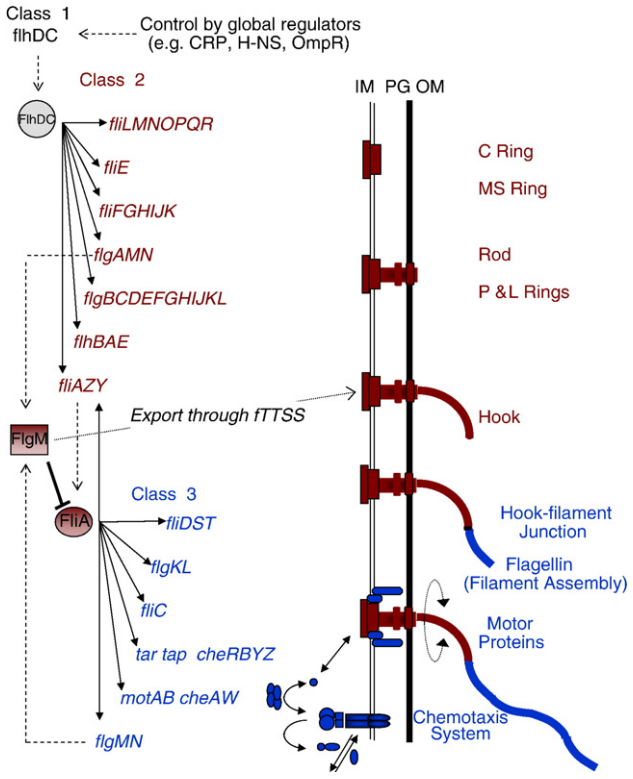

Fig. 1. Temporal order of gene expression and assembly of the flagellar apparatus. The operons for the flagella systems are expressed in a defined temporal order arranged in this figure the top to bottom [98]. The Class 2 and 3 operons and corresponding products are indicated in red and blue, respectively. The inner membrane (IM), peptidoglycan (PG) and outer membrane (OM) are as labeled. The anti-sigma factor FlgM inhibits the Class 3 sigma factor FliA until completion of a function fTTSS whereupon it gets exported from the cell. FliA also regulates its own expression [99] and contributes to the expression of other Class 2 operons [94,100].

and environmental inputs controlling the synthesis of flagella act through this operon [91–97]. Class 2 genes include structural components of the hook-basal body structure, as well as the transcriptional activator for class 3 operons. Class 3 includes flagellar filament structural genes and the chemotaxis signal transduction system that directs the cells' motion. A checkpoint mechanism ensures that class 3 genes are not transcribed before functional hook-basal body structures are completed [87,90]. This results in temporal control of gene expression allowing transcription of the final set of genes only when a macromolecular structure (the basal body) reaches an appropriate stage of assembly. This regulation of gene expression is achieved by means of export of an anti-sigma factor for the late genes (FlgM) through the completed basal body. This ensures that the filament protein is not produced until an intact fTTSS system is assembled and the two motor proteins (MotA and MotB) along with the sensory apparatus that controls the direction of flagella rotation (the chemotaxis proteins) are not made until there are mature flagella to rotate and control.

More detailed analysis of the expression of the flagella operons using promoter–reporter fusions revealed a thoroughly coordinated temporal organization of transcription [98]. Significantly, the order of expression correlated with the order of assembly into the macromolecular structure such that components required in the assembly of the flagellar complex are synthesized as required. This supply chain management optimizes the efficiency of assembly of this intricate nanomachine.

8. Conclusion

The protein interactions of the bacterial flagellum represent the highest degree of elegance in organization to facilitate the impressive function of the structure. The flagellated bacteria employ only a handful of different protein subunits that are able to self assemble into one of the most remarkable organelles known. As the flagellum represents such an ancient structure, it is without doubt that nature strongly selected for motility from the beginning, and prokaryotes that were motile were at an unquestionable advantage relative to their stationary counterparts.

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